GASPing for Life in Stationary Phase

Minireview

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Microbial Differentiation in Response to Starvation

For metazoans cellular differentiation starts as part of the developmental programs set in motion by the formation of a zygote, but for unicellular microorganisms differentiation is almost invariably a response to starvation. While supplies of essential nutrients are plentiful, microbial populations grow exponentially, but when one or more of these nutrients is exhausted growth necessarily slows down and the population enters the so-called "stationary phase." Starvation results in profound developmental changes, perhaps the most dramatic example of which is the generation of dormant spores by many species of gram-positive bacteria. In contrast, many gram-negative bacteria, among them Escherichia coli, respond to starvation by developing increased resistance to different environmental stresses without becoming dormant. These cells maintain a low level of metabolism which, under the right conditions, allows the cells to remain viable for many years as well.

Starved E. coli cells are much smaller than their rapidly growing counterparts, their cell wall is more highly cross-linked, their cytoplasm is condensed, and their periplasmic volume is increased (Huisman et al., 1996). The cells develop increased resistance to the adverse effects of high osmolarity, oxidative agents, and high temperature. These changes result from the induction of a large set of genes at the onset of starvation. This regulon is under the control of a starvation-specific transcription factor, σ^S (the product of the *rpoS* gene), which confers new promoter recognition properties on the RNA polymerase (Loewen and Hengge-Aronis, 1994). The cell regulates the level of σ^S activity at many levels: transcript abundance, efficiency of translation, and protein stability (Figure 1). Some of the small metabolites that mediate this regulation have been identified. Intracellular levels of ppGpp and cAMP regulate the levels of *rpoS* mRNA, and extracellular weak acids such as acetate and benzoate also influence *rpoS* transcription. UDP-glucose appears to be involved as a signal that represses *rpoS* translation during growth (Bohringer et al., 1995). Recently, a regulatory protein (SprE/RssB) and the ClpPX protease have been implicated in regulating the stability of σ^S, but the metabolic signals involved have not been identified (Muffler et al., 1996; Pratt and Silhavy, 1996; Schweder et al., 1996).

Metabolites Involved in Sensing Starvation and Cell Numbers

Another small metabolite, homoserine lactone (HSL), affects the levels of σ^S in the cell (Huisman and Kolter, 1994; Figure 2). The participation of HSL in the starvation response of E. coli is intriguing because structurally related compounds, acylated HSLs (Figure 2), act as extracellular signals in sensing microbial population densities, earning them the designation of "quorum-sensing" molecules (Fuqua et al., 1994). While they are now recognized as players in widely used mechanisms for sensing cell numbers among gram-negative bacteria, the role of acylated HSLs as signal molecules was first discovered in the studies of the high cell density induction of bioluminescence in the marine bacterium Vibrio fischeri. The machinery for luminescence is composed of luciferase and the enzymes that produce a fatty aldehyde that is oxidized with the concomitant release of light. Regulation of the transcription of the genes encoding these enzymes is the key process that results in cell density–dependent production of light. There are two key regulatory proteins involved: LuxR is an apo-activator of the luciferase genes and requires 3-oxo-hexanoyl-HSL as a coactivator. LuxI, encoded by a gene cotranscribed with the luciferase genes, is in turn required for the synthesis of 3-oxo-hexanoyl-HSL. At low cell densities, there is only low level transcription of the operon encoding LuxI and luciferase and thus only small amounts of LuxI to make 3-oxo-hexanoyl-HSL. Because of the acyl side chain, this molecule diffuses rapidly across membranes, and its concentration is therefore a function of the density of cells in a culture. Once enough 3-oxo-hexanoyl-HSL is made, it can bind LuxR, generating an activator that induces transcription of the operon, resulting in the increased synthesis of 3-oxo-hexanoyl-HSL. This in turn will lead to further increase in the transcription of the operon. The result is that once cells reach a critical density in culture there is a logarithmic increase in the expression of these genes. Because of their positive effect on their own synthesis, acylated HSLs are often referred to as autoinducers.

What is the connection between HSL as a signal for σ^S regulation and acylated HSLs as cell density sensors? The evidence linking HSL to σ^S levels is derived from studies with mutants in the threonine and methionine biosynthetic pathways (Huisman and Kolter, 1994; Figure 3). Mutants blocked in steps prior to the synthesis of homoserine (HS) do not induce σ^S unless they are

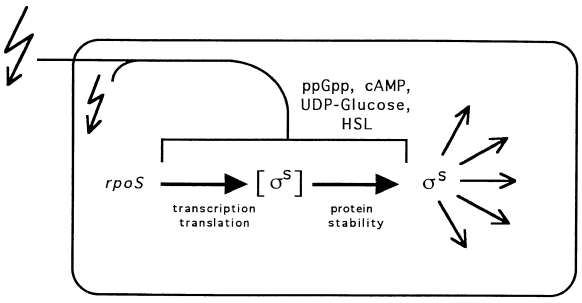

Figure 1. Signals Affecting the Regulation of σ^S Expression

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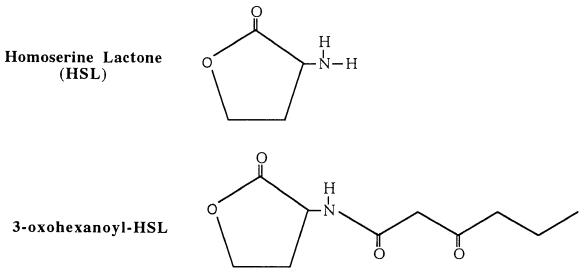

Figure 2. HSL and 3-Oxo-Hexanoyl-HSL

The acyl side chain is highly variable among different autoinducers and confers species specificity. The molecule shown is the autoinducer of bioluminescence in V. fischeri.

provided with HS or HSL. However, such mutants are competent to synthesize acylated HSLs. These mutants must be provided with threonine and methionine for growth and thus can make S-adenosylmethionine, known to be the substrate in the synthesis of acylated HSLs (Eberhard et al., 1991; More et al., 1996; Schaefer et al., 1996). Therefore, while they are structurally related, HSL and acylated HSLs appear to be produced from different metabolites. Acylated HSLs are extracellular signals whose concentration increases as a function of cell number. In contrast, HSL (derived from HS) could be an intracellular signal produced in response to starvation. We speculate that the cyclization of HS into HSL might be catalyzed by tRNA synthetases that become available owing to imbalances in amino acid pools during starvation. Several tRNA synthesases bind, adenylate, and cyclize HS to generate HSL in vitro (Jakubowski and Goldman, 1992). This suggests that a primary metabolic signal can be generated in response to starvation without requiring any changes in gene expression. Despite the different origins of the signaling molecules, there does appear to be a connection between the HSL-dependent starvation response and the acylated HSL–dependent cell number response. Mutants blocked in HS biosynthesis can make acylated HSL, but are unable to respond to it unless they are provided with HS or HSL (H. Goodrich-Blair and R. K., unpublished data). This suggests that in addition to sensing the extracellular concentration of acylated HSL,

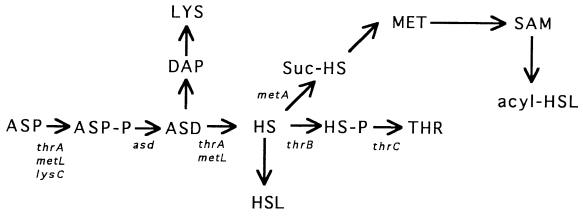

Figure 3. Schematic of the Threonine and Methionine Biosynthetic Pathways

ASP, aspartic acid; ASP-P, aspartyl phosphate; ASD, aspartate semi-aldehyde; DAP, diaminopimelate; LYS, lysine; HS, homoserine; HSL, homoserine lactone; Suc-HS, succinyl homoserine; HS-P, homoserine phosphate; MET, methionine; SAM, S-adenosylmethionine; THR, threonine. Note that not all intermediates are shown and that some of the arrows represent several steps.

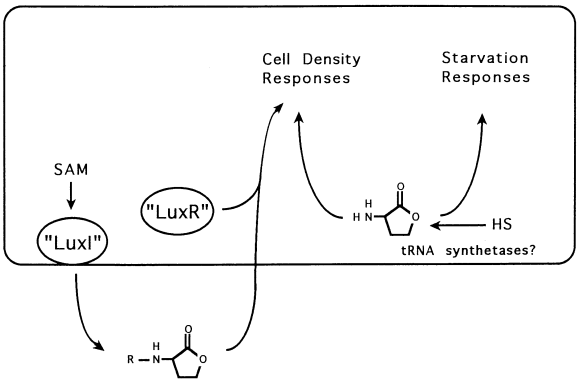

Figure 4. Speculative Model for the Involvement of HSL in Starvation-Dependent and Cell Density–Dependent Responses

See text for details.

the autoinduction circuitry also senses intracellular HSL (Figure 4).

Population Dynamics in Stationary Phase

The ability to sense starvation, generate starvation signals, and respond with changes in gene expression results in cellular differentiation and the development of increased resistances. In this way, microbial populations enter stationary phase and adapt to conditions of low nutrient availability. In standard rich medium, an E. coli culture saturates at about 10^{10} cells per milliliter, and after 1 day of incubation the population is homogeneous: virtually 100% of those cells have induced the starvation response, remain viable, and are able to restart growth if fresh nutrients are provided. However, if incubation in stationary phase is allowed to continue for several days, what ensues is anything but stationary. Despite induction of the *rpoS* regulon, prolonged starvation leads to the death of many cells in the culture. The population becomes very heterogeneous, 90%–99% of the cells die, and the few surviving cells display different morphologies (Zambrano et al., 1993). The dynamic nature of this phase of growth is apparent in the micrograph shown in Figure 5. The cells in a 10-day-old culture

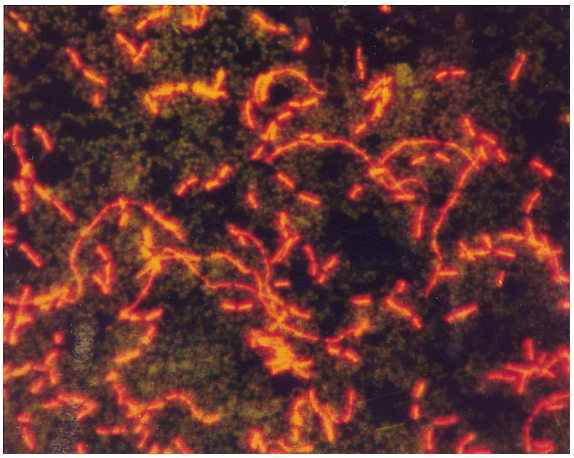

Figure 5. Micrograph of an Aztreonam-Treated 10-Day Culture of E. coli Stained with Acridine Orange

See text for details.

were treated with aztreonam, an antibiotic that inhibits septation but not cell wall elongation, and so dividing cells appear as "snakes." Cells were stained with acridine orange, making dead cells appear green and live cells orange. Among the surviving cells there are several subpopulations: some cells divide rapidly, while others do not.

Are some of the survivors in a 10-day-old culture physiologically different from 10-day-old cells? What allows some cells to divide after 10 days of incubation without added nutrients? Through the design of a mixed culture experiment it was possible to examine whether the cells from 10-day-old cultures were physiologically different from 1-day-old starved cells (Zambrano et al., 1993). Cells from 10-day-old cultures were inoculated as a minority into a 1-day-old culture. By marking the otherwise isogenic strains with different selectable alleles, it was possible to follow the fate of the cells derived from each culture. Surprisingly, members of the minority population derived from the 10-day-old cultures grew, while the cells derived from the 1-day-old culture died off completely. Cells from older cultures had a growth advantage in stationary phase (GASP) phenotype. Cells expressing the GASP phenotype are most probably able to grow because they compete better for nutrients released by the dying cells from the majority population. The GASP phenomenon has been observed with many strains and under many conditions and may be a commonly used strategy for microbial survival during prolonged starvation.

The GASP Phenotype Can Result from Mutations in rpoS

Analysis of cells expressing the GASP phenotype revealed that this phenotype was due to mutations and not to a reversible physiological adaptation. Some of the mutations that confer the GASP phenotype are found in *rpoS*. Alleles of *rpoS*, designated *rpoS*^{att}, that reduce activity of the transcription factor in stationary phase but do not abolish it completely are often selected for when a wild-type strain is incubated for 10 days. Transduction of these *rpoS*^{att} alleles into wild-type cells is sufficient to express the GASP phenotype in mixed cultures.

It is so far unclear how decreased σ^S activity can confer on cells a GASP phenotype. However, given that *rpoS*^{att} mutations give cells a growth advantage in stationary phase, it is interesting that not all laboratory strains carry such mutations. The *rpoS*^{att} mutations that confer the GASP phenotype also make cells more sensitive to different environmental assaults and thus are likely to be selected against under other conditions. Nonetheless, many laboratory strains and natural isolates of E. coli show allelic variation in *rpoS*, suggesting that rapid population takeovers occur commonly in the laboratory and in the natural environment.

Evolution in Stationary-Phase Cultures

Figure 6 depicts what probably occurs during prolonged incubation of cultures in stationary phase. An advantageous GASP mutation may arise in the population, which allows these mutants to utilize more efficiently nutrients released by the dying cells. As a result, the original population is rapidly replaced by this fitter strain. Most importantly, additional incubation can bring about successive rounds of selection in which new cells with additional GASP-conferring mutations arise and result in new population changeovers.

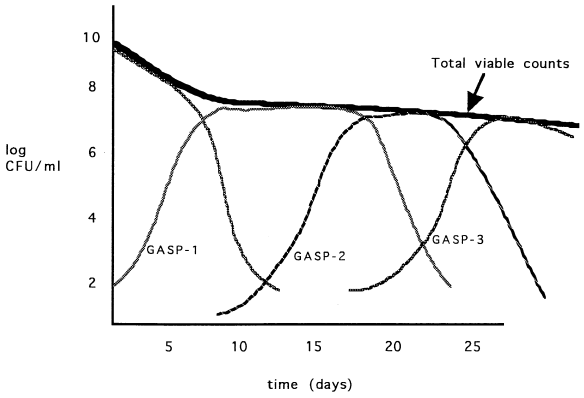

Figure 6. Theoretical Growth Curve Representing Successive Takeovers by GASP Mutants in a Stationary-Phase Culture

See text for details.

The discovery of the GASP phenotype has important implications for the study of the origin of mutations in starved microorganisms. Several studies suggest that mutations that confer an adaptive advantage occur at increased rates in nondividing cells (Foster, 1993; Rosenberg, 1994). These "postselection" mutations have been assumed to occur in static or nearly static stationary-phase colonies or cultures. But GASP mutants are able to grow as a minority population when most cells are dying in the absence of fresh nutrients, suggesting that some postselection mutations may occur in a minority of mutant cells growing slowly under conditions of starvation.

Stationary-phase cultures are dynamic and can undergo population shifts. The composition of the population in the culture changes as fitter cells grow and take over the population. This is reminiscent of the population changes that occur during continuous growth of E. coli cultures known as periodic selections (Dykhuizen and Hartl, 1983). However, the GASP takeovers observed in stationary-phase cultures occur much more rapidly than the population shifts observed in continually growing cultures, where the fraction of mutants increases gradually, and they take over only after prolonged growth involving hundreds to thousands of generations. The GASP phenotype reflects the remarkable versatility of bacteria to respond to conditions of extreme starvation.

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